Journal of Applied Biosciences 49: 3383– 3387

ISSN 1997–5902

Cytosine DNA methylation changes drought stress responses in tissue culture derived banana (*Musa* AAA – East Africa) plants

Msogoya T.J[1*] and Grout B. W [2]

[1]Department of Crop Science and Production, Sokoine University of Agriculture, P.O Box 3005, Morogoro, Tanzania
[2]Life Sciences, University of Copenhagen, 2630-Taastrup, Denmark

*Corresponding author - E-mail: tjmsogoya@suanet.ac.tz or tjmsogoya@yahoo.com
Originally Submitted on 18th October 2011. Published online at www.m.elewa.org on January 29, 2012.

ABSTRACT

Objective: Tissue culture derived plants are often vulnerable to abiotic stresses but mechanisms underlying such responses are hardly known. This study was conducted to determine mechanisms underlying drought stress vulnerability of *in vitro* derived banana cv. 'Uganda'.

Methodology and results: Suckers of *in vitro* derived off-type, *in vitro* micropropagation (MP) derived normal plants and conventionally propagated (CP) plants with no tissue culture history in their ancestry were collected in the field at Sokoine University of Agriculture and planted in 20-litre containers for drought stress evaluation. The mechanisms underlying the drought stress vulnerability were determined when banana plants reached 1.5 m tall based on leaf global cytosine DNA methylation, stomatal density and leaf senescence. Global cytosine DNA methylation was determined from cigar leaves by a reversed phase HPLC analysis. Leaf stomatal density was determined as the number of stomata per unit area of both upper and lower leaf surfaces. Leaf senescence was estimated as a number of leaves with dying margins when soil moisture level decreased to minus 630 millibars. The off-type and MP derived plants had lower ($P < 0.05$) global cytosine DNA methylation of 11.3 and 17.5 % compared with 22.5 % of the CP derived plants. On the contrary, the off-type and MP derived plants had higher stomatal density of 78.2 and 78.8 stomata per mm^2 on the lower leaf surface compared to 72.0 stomata per mm^2 of the CP derived banana plants. The leaf senescence of the off-type and MP derived plants was significantly ($P < 0.05$) higher with 87.7 and 79.5 % compared to 66.7 % of the CP derived plants at soil moisture of minus 630 millibars.

Conclusion and application: These findings provide evidence that tissue culture process increases the vulnerability to water stress of *in vitro* banana regenerants as a consequence of increased leaf stomatal density which is possibly under the control of cytosine DNA hypomethylation. The vulnerability of the *in vitro* derived banana cv. 'Uganda' limits the use of tissue culture derived planting materials among small-scale farmers with limited water resource and irrigation facilities but provides an opportunity for further studies to minimise water stress susceptibility of *in vitro* derived banana suckers.

Key words: DNA methylation, Stomatal density, Drought stress, *In vitro* derived banana

INTRODUCTION

Explant preparation and the use of growth regulators to induce regenerative competence during plant *in vitro* micropropagation are stressful to plant cells (Cassels and Curry, 2001). This stress triggers production of toxic free radicals and non-radical reactive oxygen species (Johnston *et al*., 2006). The

toxic substances can attack nucleic acids, resulting in the production of genotoxic products such as 5'-methyl-2-deoxycytidine (Bialkowski and Olinski, 1999; Benson, 2000). The formation of 5'-methyl-2-deoxycytidine is known as cytosine DNA methylation and this product alters the ability of RNA polymerase and transcriptional proteins to bind to the DNA during transcription (Slater *et al*., 2003), resulting in epigenetic gene expression such as silencing and repression (Kazmierczak, 1998; Slater *et al*., 2003). The frequently encountered somaclonal variation among tissue culture-derived plant regenerants has been associated with DNA methylation (Kaeppler *et al*., 2000; Cassels and Curry, 2001). DNA methylation affects the way plants adjust to the environment, including responses to coldness (Deumling and Clermont, 1989). The number and function of stomata are influenced at an early stage of plant development by the environmental conditions (Jonathan *et al*., 1997; Majada *et al*., 2001). Humid *in vitro* culture conditions coupled with limited gaseous exchange in airtight culture vessels affected stomatal development and guard cell functioning (Campos *et al*., 2003). For instance, carnation plantlets propagated under humid culture conditions had higher stomatal density with stomata failing to close properly when subjected to darkness, abscissic acid and polyethylene glycol (Majada *et al*., 1998). The changes in stomatal density and closure affect the survival of *in vitro* plantlets not only during acclimatisation but also after field establishment (Kirdmanee *et al*., 1996). On the other hand, the aseptic conditions during *in vitro* propagation results in mass production of pest-free *in vitro* plants but also removes endophytes required for natural plant defence against biotic and abiotic stresses, including drought stress (Hamill *et al*., 2005).

Field grown *in vitro* derived off-type and true-to-type plants of East African banana cv. 'Uganda' exhibited higher drought stress vulnerability than the conventionally derived banana plants with no tissue culture history in their ancestry. The mechanisms underlying this vulnerability are hardly known. The objective of this study was to determine the mechanisms underlying the drought stress vulnerability of *in vitro* derived off-type and true-to-type banana cv. 'Uganda' based on leaf cytosine DNA methylation and stomatal density.

MATERIALS AND METHODS

Description of study areas and plant materials: Sword suckers were collected from the field-grown parent banana cv. 'Uganda'. The suckers were planted in 20-litre containers in February 2007 at Sokoine University of Agriculture, Tanzania. The plant growth medium consisted of forest soil, farmyard manure and rice husk at 3:2:1 (v/v), respectively. The container plants were raised under a polyethylene plastic tunnel with average temperatures and relative humidity of 26 °C and 68 %, respectively.

Experimental design: The experimental setup consisted of a complete randomised block design with three treatments. These treatments were *in vitro* originated off-type suckers at the fourth ratoon cycle (treatment 1), *in vitro* originated normal suckers at the fourth ratoon cycle (treatment 2) and conventional propagated suckers with no tissue culture history in their ancestry (control). Each treatment was replicated three times and a replicate consisted of 10 plants.

Cytosine DNA methylation and stomatal density: The DNA methylation and stomatal density were determined from cigar leaves before withholding irrigation. Ten cigar leaves of off-type, MP and CP derived banana plants at 1.5 m tall were collected for determination of DNA methylation and stomatal density. Nucleic acids for determination of DNA methylation were extracted using CTAB-based procedure with modifications according to Ramage *et al.* (2004). The nucleic acids were digested into nucleotides and nucleosides using nuclease P1 (Sigma N-8630) and bacterial alkaline phosphatase (Sigma P-4252), respectively (Chakrabarty *et al*., 2003; Johnston *et al*., 2005). Nucleoside chromogram was generated using a reversed phase HPLC and the percentage global cytosine DNA methylation was calculated according to Johnston *et al.* (2005) as follows:

$$mDNA = 100 * \frac{[mdC]}{[dC + mdC]}$$

Where, *m*DNA*:* Cytosine DNA methylation (%), *mdC*: Methylated DNA cytosine (μM) and *dC*: Non-methylated DNA cytosine (μM).

The cigar leaf stomatal density was determined according to Marin *et al.* (1988). Stomatal density was calculated as the number of stomata per unit area of the leaf upper and lower surfaces.

Plant physiological responses to drought stress: The off-type, MP and CP derived plants at about 1.5 m tall stage were water-stressed by withholding irrigation until the soil moisture level monitored by a tensiometer (Eijkelkamp Agrisearch, Netherlands) reached minus 630 millibars. As 80 % of banana pseudostem constitutes water, the plant response to water stress was assessed based on reduction in pseudostem diameter and leaf senescence. Ten plants for each replicate were randomly selected for measurements of pseudostem diameter. The diameter was measured at a height of 60 cm from the root collar using veneer a calliper. All leaves in a plant were used for drought stress assessment. The leaf senescence was estimated based on the number of leaves per plant with dying margins.

Data analysis: Percentage data were arcsine-transformed prior to analysis using SPSS 15.0 computer statistical programme (SPSS, 2006). The data were subjected to analysis of variance, and multiple means comparison was performed based on Tukey honest significant difference (Tukey-HSD) test ($P < 0.05$) (Zar, 1997).

RESULTS AND DISCUSSION

The off-type and MP derived plants had significantly ($P < 0.05$) lower global cytosine DNA methylation of 11.3 and 17.4% compared with 22.5 % of the CP derived banana (Table 1). The reduction in cytosine DNA methylation in the off-type and MP derived plants possibly occurred during the *in vitro* induction of adventitious shoots. Several studies have reported DNA hypomethylation during callus induction in many plant species (Harding *et al*., 1996; Koukalova *et al*., 2005). For instance, cytosine DNA methylation decreased from 44.0 % in the donor plants to 32.8 % in the callus-derived regenerants of Siberian squill plants (Deumling and Clermont, 1989).

Table 1: Leaf cytosine DNA methylation, stomatal density and senescence of drought stress vulnerable *in vitro* regenerants of banana cv. 'Uganda'

Variable	Off-type	MP plants	CP plants
Cytosine DNA methylation (%)	$11.3^{a} \pm 0.5$	$17.4^{b} \pm 0.3$	$22.5^{c} \pm 1.3$
Number of stomata/mm^2 (Lower face)	$78.2^{b} \pm 1.3$	$78.8^{b} \pm 1.5$	$72.0^{a} \pm 1.0$
Number of stomata/mm^2 (Upper face)	$16.0^{b} \pm 0.3$	$12.3^{a} \pm 0.2$	$11.0^{a} \pm 0.1$
Leaf senescence per plant (%)	$87.7^{C} \pm 3.3$	$79.5^{b} \pm 4.4$	$66.7^{a} \pm 5.1$
Reduction in pseudostem diameter (%)	$17.4^{b} \pm 0.5$	$16.0^{ab} \pm 0.6$	$14.9^{a} \pm 0.9$

Mean bearing the same superscript letter within the row are not significantly ($P < 0.05$) different according to Tukey-HSD test.

Changes in DNA methylation during *in vitro* culture are influenced by stress, especially that caused by growth regulators used to induce tissue regenerative competence (Koukalova *et al*., 2005). For example, the loss in DNA methylation increased with the amount of kinetin in the growth media in callus-derived carrot regenerants (Arnholdt-Schmitt *et al*., 1991). A reduction in DNA methylation has been associated with cold stress treatment in *Arabidopsis* (Finnegan *et al*., 1998) and maize seedlings (Steward *et al*., 2002). The increased drought stress vulnerability of the *in vitro* derived banana cv. 'Uganda' possibly involved a loss in cytosine DNA methylation. Losses in cytosine DNA methylation have similarly been associated with vulnerability to coldness in callus-derived Siberian squill plants (Deumling and Clermont, 1989). The leaf lower surface of the off-type and MP derived plants had significantly ($P < 0.05$) higher stomatal density of 78.2 and 78.8 stomata per mm^2 than the CP derived plants with 72.0 stomata per mm^2 (Table 1). High leaf stomatal densities have also been reported in several tissue culture derived plant species, including bamboo and apple (Blanke and Belcher, 1989). The increased stomatal density of the *in vitro* derived banana encountered in this study was possibly caused by tissue culture conditions, especially high humidity in airtight culture vessels. An increased stomatal density has also been reported in tissue culture derived carnation plants (Majada *et al*., 1998). Correspondingly, the off-type and MP derived banana plants had significantly ($P < 0.05$) higher leaf senescence of 87.7 and 79.5 % compared with 66.7 % of the CP derived plants (Table 1). The deterioration of field-grown *in vitro* derived plants as a result of excessive water loss has also been reported in several plant species (Marin *et al*., 1988; Kirdmanee *et al*., 1996).

CONCLUSION

The increased drought stress vulnerability of field-grown *in vitro* derived regenerants of banana cv. 'Uganda' is due to high stomatal density, which is under the control of cytosine DNA hypomethylation. This is the first report showing the association between cytosine DNA hypomethylation and drought stress vulnerability in tissue culture derived banana plants. The water stress vulnerability of *in vitro* derived banana plants may limit the use of tissue culture derived planting materials among small-scale farmers who entirely rely on the unreliable rainfall for banana production. Further studies are required to minimise water stress vulnerability of *in vitro* derived planting materials before their distribution to farmers.

ACKNOWLEDGEMENT

Authors gratefully acknowledge the PANTIL Programme of Sokoine University and Commonwealth Scholarship Commission of the UK (Scholarship Ref. TZA-2004-129) for financing this study.

REFERENCES

Arnholdt-Schmitt B, Holzapfel B, Schillinger A. and Neumann KH: 1991. Variable methylation and differential replication of genomic DNA in culture of carrot root explants during growth induction as influenced by hormonal treatments. *TAG Theoretical and Applied Genetics* 82. www.springerlink.com/content/p260083536337 mhw.

Benson EE: 2000. Special symposium: *In vitro* plant recalcitrance - Do free radicals have a role in plant tissue culture recalcitrance? *In Vitro Cellular and Developmental Biology – Plants* 36: 163 – 170.

Bialkowski K. and Olinski R: 1999. Oxidative damage to plant DNA in relation to growth conditions. *Acta Biochimica Polonica* 46: 43 – 49.

Blanke MB and Belcher AR: 1989. Stomata of apple leaves cultured *in vitro. Plant Cell Tissue Organ Culture* 19: 85 – 89.

Campos PS, Quartin V, Ramalho J and Nunes MA: 2003. Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. *Journal of Plant Physiology* 160: 283 – 292.

Cassells AC and Curry RF: 2001. Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. *Plant Cell, Tissue and Organ Culture* 64: 145 – 157.

Chakrabarty D, Yu KW and Peak KY: 2003. Detection of DNA methylation changes during somatic embryogenesis of Siberian ginseng (*Eleuterococcus senticosus*). *Plant Science* 165: 61 – 68.

Deumling B. and Clermont L: 1989. Changes in DNA content and chromosome size during cell culture and plant regeneration of *Scilla siberica*: selective chromatin diminution in response to environmental conditions. *Chromosoma* (Berl) 97: 439 – 448.

Finnegan EJR, Genger K, Kovac K, Peacock WJ and Dennis ES: 1998. DNA methylation and the promotion of flowering by vernalisation. *The National Academy of Sciences*. http://www.pnas.org/content/95/10/5824.abstr act.

Hamill S, Wasmund K, Smith M, Eccleston K and Mackay D: 2005. Endogenous bacteria isolated from banana meristem during tissue culture initiation: problems and potential, in *Proceedings of the Australian Branch of the IAPT and B*, Perth, Australia, 21 -24th September 2005, 101 – 111.

Harding K, Benson EE, Kaliope A and Roubelakis-Angelakis AK: 1996. Methylated DNA changes associated with the initiation and maintenance of *Vitis vinifera in vitro* shoot and callus cultures: A possible mechanism for age-related changes. *Vitis* 35: 79 – 85.11

Johnston C, Day JD, Staines H and Benson EE: 2006. An *in vitro* oxidative stress test for determining pollutant tolerance in algae. *Ecological indicators* 6: 770 - 779.

Johnston JW, Harding K., Bremner DH, Souch G, Green J, Lynch PT, Grout BWW and Benson EE: 2005. HPLC Analysis of DNA Methylation in Plants. *Plant Physiology and Biochemistry* 43: 844 – 853.

Jonathan D, Weyers B and Lawson T: 1997. Heterogeneity in stomata characteristics. *Advances in Botanical Research* 26: 317 – 352.

Kaeppler S, Kaeppler HF, and Rhee Y: 2000. Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology* 43: 179 – 188.

Kazmierczak J: 1998. Effect of DNA methylation on potential transcriptional activity in different tissues and organs of *Vicia faba* spp. minor. *Folia Histochemistry and Cytobiology* 36: 45 – 49.

Kirdmanee C, Kozai T, and Adelberg J: 1996. Rapid acclimatisation of *in vitro* eucalyptus plantlets by controlling relative humidity *ex vivo*. *Acta Hortic.* 440: 616 - 621.

Koukalova B, Fojtova M, Lim KY, Fulnecek J, Leitch AR and Kovarik A: 2005. Dedifferentiation of tobacco cells is associated with ribosomal RNA gene hypomethylation, increased transcription and chromatin condensation alterations. *Plant Physiology* 139: 275 – 286.

Majada JP, Centeno ML, Feito I, Fernandez B and Sanchez-Tames R: 1998. Stomatal and cuticular traits on carnation tissue culture under different ventilation conditions. *Plant Growth Regulators* 25: 113 – 121.

Majada JP, Sierra MI and Sanchez-Tames R: 2001. Air exchange rate affects the *in vitro* developed leaf cuticle of carnation. *Scientia Horticulturae* 87: 121 – 130.

Marin JA, Gella R and Herero M: 1988. Stomatal structure and functioning as a response to environmental changes in acclimatised micropropagated *Prunus ceracus* L. *Annals of Botany* 62: 663 - 670.

Ramage CM, Borda AM, Hamill SD and Smith MK: 2004. A simplified PCR test for early detection of dwarf off-types in micropropagated Cavendish bananas (*Musa* spp. AAA). *Scientia Horticulturae* 103: 145 – 151.

Slater A, Scott N and Folwer M: 2003. Plant Biotechnology: Gene manipulation in plants. Oxford University Press, New York.

SPSS^{R} 2006. Statistical package for the social sciences (SPSS) (Version 15.0). SPSS Inc., Chicago.

Steward N., Ito M., Yamaguchi Y., Koizumi N., and Sano H: 2002. Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress. *Journal of Biological Chemistry* 277: 37741 – 37746.

Zar JH: 1997. Biostatistical analysis (3rd Edition). Entice-Hall International Inc., Upper Saddle River.